THE AGGLUTINATION OF FISH ERYTHROCYTES BY NORMAL HUMAN SERA

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The discovery that normal human sera strongly agglutinate the erythrocytes of some species of fish led the authors to investigate this phenomenon systematically. This study represents part of a general program (Cushing, 1952a, 1952b) intended to develop serological techniques that might be useful in making racial and evolutionary studies on fish similar to those being made on man (Race and Sanger, 1950) and such other animals as doves (Irwin, 1947) and cattle (Stormont, Owen and Irwin, 1950).

Although Nuttall (1904) included fish in his classical work on the serological differentiation of animal bloods, relatively little has since been done with the serology of this group of vertebrates. The references known to these authors are listed in the bibliography or may be found by consulting the papers given. These include work by the following: Streng, 1938; Cushing, 1942, 1945a, 1945b; Gemeroy, 1943; Jonsson, 1944; Tyler and Metz, 1945; Tyler, 1946; and Grubb, 1949.

In addition, attention is called to the work of Cotterman (1944) who used normal human serum to demonstrate differences in mice of the genus *Peromyscus* and to that of Tamm (1952) demonstrating species differences in the viral agglutination of fish erythrocytes.

In order to establish a point of departure for comparative study, two species of marine fish were selected, the shiner sea-perch, *Cymatogaster aggregata* (Gibbons), a member of the viviparous perches (Embiotocidae), and the kingfish, *Genyonemus lineatus* (Ayres), a croaker (Sciaenidae). These were chosen primarily because their red blood cells were strongly agglutinated by human sera and because of the ease with which they could be captured locally and kept alive in the laboratory.

Materials and Methods

The physiological saline used was prepared by diluting filtered sea water with an equal volume of distilled water. This gave a final concentration of approximately 1.5% sodium chloride. The use of sea water avoided the possibility of hemolysis induced in the cells of some species of fish by some artificially prepared sodium chloride solutions (Ball, 1933). The agglutination of human erythrocytes was not affected by the use of this saline.

Fish erythrocytes were obtained by heart puncture. Blood was drawn into syringes containing an approximately equal volume of heparin solution. This solution had a concentration of 50 mg. of heparin for every 10 ml. of physiological saline. This preparation was found to be satisfactory for most of our work, but further re-

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search on the usefulness of other concentrations of heparin and on other kinds of anti-coagulants is desirable.

Erythrocytes were washed free of serum proteins in physiological saline and finally suspended in a concentration approximating two per cent cells.

The human sera used in these studies were obtained either from local individuals, in which case their complement was heat-inactivated before use, or in the case of blood typing sera, from the Cutter Laboratories, Berkeley. The particular sera used in an experiment are referred to in the legends of the tables presenting the data for that experiment. Sera were kept frozen when not being used, inasmuch as repeated freezing and thawing were not observed to have any effect upon the agglutinins being studied.

Slide agglutination tests were used to determine the presence or absence of the various antigens and antibodies studied. These tests were made at room temperature by placing single drops of the appropriate cell suspensions and sera together and mixing these by hand rotation of the slide. Readings were made by eye at the end of 15 minutes without the aid of a microscope and checked at 30 minutes. Drying was prevented by placing Petri dish covers over the slides.

Absorptions of sera by erythrocytes were made by placing 0.1 ml. of packed cells in small test tubes with 1.0 ml. of serum and either rotating these mixtures at a nearly horizontal angle by attaching the tubes to a disc turned on a stirring motor, or by placing the tubes in a horizontal position and shaking them through their long axes on a Yankee-Kahn Shaker. In this later procedure, a small glass bead was added to each tube. The time of absorption varied in individual cases from 5 to 20 minutes. In general it was found that a five-minute absorption on the shaker was sufficient to obtain suitable preparations.

These preparations were obtained with a view of decreasing the titers of the antibodies being absorbed relative to those not being absorbed to a point where the slide test gave negative readings of the absorbed antibody while showing that the titers of unabsorbed antibody remained the same. While the slide test was entirely adequate for the studies reported here, it has been suggested that the test tube method (Schiff and Boyd, 1942; pp. 31–33), enhanced by centrifugation, may prove to be a faster and more sensitive test for future work.

Care was taken to insure that all the sera used were sterile and therefore did not contain agglutinins produced by bacteria.

Results

The antigens described below represent the minimum number of antigenic differences that can be detected between *Cymatogaster* and *Genyonemus* cells. The possibility exists that a single antigenic entity may be resolved into two or more components with the discovery of suitable antibodies. The F-3 and F-4 antigenic components described below were thus resolved.

The results of studies of the sera of several individuals, which were collected at random, indicate that the titers of red cell agglutinins for fish and humans vary independently. The indicated titers (Table I) should be taken as reasonable approximations only, inasmuch as it is possible that individual variations in red cells might influence the relative strength of the reactions to some extent (Wiener, 1943; p. 20). The agglutinins involved are stable to heating at 56° C. for 25 minutes. That dif-

TABLE I

Agglutination of fish and human erythrocytes by normal human sera

Serum from human type	Cells from	Serum dilutions				
		1/4	1/8	1/16	1/32	1/64
AB	*Cymatogaster*	3+	+	±	0	0
	Genyonemus	0	0	0	0	0
AB	*C.*	+	±	0	0	0
	G.	0	0	0	0	0
A	*C.*	2+	+	0	0	0
	G.	±	0	0	0	0
	B (Human)	±	0	0	0	0
A	*C.*	4+	4+	3+	2+	+
	G.	±	0	0	0	0
	B H.	±	±	0	0	0
B	*C.*	4+	3+	3+	±	0
	G.	2+	±	0	0	0
	A H.	+	0	0	0	0
B	*C.*	2+	+	0	0	0
	G.	0	0	0	0	0
	A H.	2+	±	0	0	0
O	*C.*	4+	4+	3+	2+	0
	G.	3+	+	0	0	0
	A H.	2+	+	±	0	0
	B H.	3+	2+	+	0	0
O	*C.*	3+	3+	+	±	0
	G.	+	0	0	0	0
	A H.	+	±	0	0	0
	B H.	+	+	±	0	0
O	*C.*	4+	3+	3+	2+	2+
	G.	2+	±	0	0	0
	A H.	3+	3+	+	0	0
	B H.	3+	+	+	0	0

The reactions of several human sera with fish and human erythrocytes are shown. All sera were from a single collection, twenty-four hours old, and were heat-inactivated at 56° C. for 25 minutes before using. Agglutination tests were run as described in the text. Degree of agglutination, estimated visually in terms of the number of cells agglutinated, is described as follows: 4+ = essentially all; 3+ = three-fourths; 2+ = one-half; + = one-fourth; ± = definite agglutination but less than one-fourth.

ferent agglutinins from the anti-A anti-B agglutinins may be involved in the reactions is suggested by the fact that the fish and the human red cell titers vary independently. Further evidence for this conclusion is obtained by the absorptions indicated in Table II. It can be seen from this table that since *Cymatogaster* cell absorption of anti-A plus anti-B sera fails to remove either anti-A or anti-B from the

pooled sera, *Cymatogaster* cells must possess at least one antigen distinct from the A and B antigens on human cells. This is at once apparent when the sera of type AB individuals are studied since these individuals lack detectable anti-A or anti-B bodies in their sera but show titers against fish red cells.

TABLE II

Differentiation of an antigen on Cymatogaster erythrocytes

Unabsorbed sera	Cells			Sera absorbed with these cells	Cells		
	Human A	Human B	*Cymatogaster*		Human A	Human B	*Cymatogaster*
Anti-A	4+	0	4+	Human A	0	0	4+
Anti-B	0	4+	4+	Human B	0	0	4+
Anti-A+B	4+	4+	4+	*Cymatogaster*	4+	4+	0

The antisera were commercial preparations derived from pooled human sera. These were used in a final dilution of 1 in 2 from that of the original preparation as received in this laboratory. The anti-A+B serum was prepared by mixing equal volumes of the anti-A and anti-B and was used without further dilution.

The reactions of *Genyonemus* cells with human typing serum are more complex (Table III). *Genyonemus* cells carry an antigenic component that is different from that of *Cymatogaster* for it resembles the human B antigen in its reactions with anti-B serum. Further evidence for this point is the fact that *Genyonemus* cells are much more rapidly and strongly agglutinated by anti-B serum than by anti-A serum.

TABLE III

Demonstration of an antigen on Genyonemus cells that is serologically related to the human B antigen

Cells	Unabsorbed serum			Absorbed with *Genyonemus* cells			Absorbed with B cells		
	1/4	1/8	1/16	1/4	1/8	1/16	1/4	1/8	1/16
Human B	4+	3+	+	+	0	0	0	0	0
Cymatogaster	4+	3+	+	4+	3+	+	4+	3+	+
Genyonemus	3+	2+	0	0	0	0	0	0	0

The serum used was obtained from a type A human and was selected because it not only contained human anti-B and *Genyonemus* red cell agglutinins, but *Cymatogaster* red cell agglutinins as well.

Genyonemus cells react as strongly with anti-A typing serum after absorption of the anti-A antibodies as before (Table IV). The *Genyonemus* cells have at least one antigen other than the B antigen on their cells. This antigen is not related to human A. This point is confirmed by the occurrence of a human AB serum that agglutinates *Genyonemus* and *Cymatogaster* cells, but not human cells of type A or B (Table V).

It is therefore concluded that *Genyonemus* erythrocytes possess one antigen very similar to the human B antigen, and a second antigen distinct from either the human A or B antigen. *Cymatogaster* can be shown to possess one antigen distinct from the human A or B antigens.

For convenience in further discussion the two *Genyonemus* antigenic components will be numbered F-1 (the B-like antigen) and F-2 (the not-B antigen).

TABLE IV

Differentiation of Genyonemus and human A antigens

Cells	Dilutions of unabsorbed anti-A			Dilutions of anti-A absorbed with human A cells		
	1/1	1/2	1/4	1/1	1/2	1/4
Human A	4+	4+	3+	0	0	0
Cymatogaster	4+	3+	±	4+	3+	±
Genyonemus	2+	±	0	2+	±	0

The antiserum used was a commercial preparation of pooled human sera and was diluted after absorption.

The *Cymatogaster* antigen will be numbered F-3. That it is distinct from F-1 and F-2 will be shown below. The letter F is used here to denote that these are antigens found on fish cells, and to give distinctiveness to the numbers used.

The differentiation of F-2 and F-3 is based upon several facts. First, the anti-B serum absorbed with *Genyonemus* cells still retained an unimpaired ability to agglutinate *Cymatogaster* cells (Table III) which they would not have done had F-2 and

TABLE V

Differentiation of an antigen on Genyonemus cells

Cells	Dilutions of unabsorbed serum				Dilutions of serum absorbed with *Genyonemus* cells			
	1/2	1/4	1/8	1/16	1/2	1/4	1/8	1/16
Cymatogaster	4+	4+	+	0	4+	4+	+	0
Genyonemus	4+	+	0	0	0	0	0	0

The antiserum used was from an AB individual selected because it agglutinated both *Cymatogaster* and *Genyonemus* cells, but failed to agglutinate human A, B or sheep red cells. The serum was diluted after absorption.

F-3 been alike antigenically. Second, the absorption with *Genyonemus* cells of the AB serum capable of agglutinating both *Genyonemus* and *Cymatogaster* cells left the *Cymatogaster* agglutinins intact (Table V).

Additional evidence shows that *Cymatogaster* and *Genyonemus* cells each have at least one antigen peculiar to themselves, although not necessarily F-2 and F-3. A rabbit anti-*Cymatogaster* serum that agglutinated *Cymatogaster* cells in dilutions

greater than 1 in 600 failed to agglutinate *Genyonemus* cells in any dilution. Also a rabbit anti-yellowfin tuna serum strongly agglutinated *Cymatogaster* cells and only weakly agglutinated *Genyonemus*. The reverse was true of an anti-skipjack serum that did not agglutinate human B cells.

Once distinguished, the F-3 (*Cymatogaster*) antigen could be further resolved into two separate antigenic components on the basis of the following observations. Sera of human AB individuals were found which agglutinated *Cymatogaster* but not sheep cells. The designation F-3 is retained for the antigen involved in this agglutination reaction.

In addition, anti-sheep cell sera were found to agglutinate strongly *Cymatogaster* cells but not those of *Genyonemus*. Absorption of rabbit anti-sheep cell serum with *Cymatogaster* cells left agglutinins for sheep cells only, but sheep cells removed both sheep and *Cymatogaster* agglutinins from this serum (unabsorbed sheep cell titer 1 in 1000, unabsorbed *Cymatogaster* titer 1 in 64). These reactions therefore reveal a second antigen on *Cymatogaster* cells, designated as F-4.

These observations, together with the fact that sheep cells are hemolysed by the action of heat-inactivated rabbit anti-*Cymatogaster* serum (titer of hemolysin = 1 in 40) in the presence of guinea pig complement, suggest that F-4 is one of the so-called Forssman antigens (Boyd, 1947).

So far, no individual antigenic variations have been detected within the two species. However, a persistent search for these was not made.

Discussion

We find that normal human sera contain at least three agglutinins that are specific for antigenic components on the erythrocytes of *Genyonemus* and *Cymatogaster*. One of these is the anti-B agglutinin, while the other two represent antibodies not related to the classical isoantibodies. Whether these antibodies are genetically determined, or are the result of immune responses to infection (Wiener, 1951), remains to be found. The three antibodies serve to identify two antigenic components on *Genyonemus* red cells, one of which is similar to the human B antigen, and one antigenic component on *Cymatogaster*.

In addition, rabbit anti-serum against sheep erythrocytes has been used to demonstrate a second antigenic component on *Cymatogaster* erythrocytes. This component resembles the Forssman antigens in its properties.

The term antigenic component is used to differentiate the two serologically distinct entities of each species for the reason that available evidence cannot differentiate between two alternative hypotheses: (1) that each serologically recognizable component of a given fish is actually a distinct and separate antigen; (2) that each serologically recognizable component of a given fish represents a difference in the reactions of two kinds of antibodies with a single antigen on the cells of that fish.

In other words, unless the antigenic components can be separated genetically (or otherwise) it is possible that *Cymatogaster* and *Genyonemus* may differ only in a minimum of two antigens rather than four. The problem involved is similar to that encountered in the study of some of the antigens in cattle (Stormont, Owen and Irwin, 1950), although the situation in cattle is, of course, much more complex, involving genetic as well as serological data.

No individual variations in the occurrence of these antigenic components have been noted in local populations of the two species of fish.

An extensive comparative study of the distribution in fishes of antigens reacting with human agglutinins and anti-sheep serum has been made and will be reported in another paper.

It is possible that the fish cell agglutinins discovered in human serum may be of clinical interest, as for example in cases of allergies to fish foods.

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Summary

1. Normal human sera were found to agglutinate the erythrocytes of the shiner sea-perch (*Cymatogaster aggregata*) and of the kingfish (*Genyonemus lineatus*).

2. Agglutination was shown to occur by virtue of four different antigens, each species having two of these four in its erythrocytes.

3. Of these, *Genyonemus* had an antigen with a specificity closely resembling the human B substance (designated here as F-1) and a species-specific antigen (F-2) peculiar to itself.

4. *Cymatogaster* had a species-specific antigen (F-3) and an antigen with a specificity resembling that of the Forssman antigen (F-4).

5. No individual variations were found in either fish with respect to these antigens.

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